

IN THE MATTER of United States
Patent Application No. 09/423093
in the name of The University of
Sydney

INVENTOR DECLARATION

I, Professor Peter Richard Reeves (Head of Microbiology Discipline in the School of Molecular and Microbial Biosciences, University of Sydney), of 20 Mansfield Street, Glebe, NSW 2037, Australia make the following declaration:

1. "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65°C at a salt concentration of 0.1xSSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, e.g., wash conditions of less than 60°C at a salt concentration of 1.0xSSC. For example, high stringency conditions include hybridization at 42°C in the presence of 50% formamide; a first wash at 65°C in the presence of 2xSSC and 1% SDS; followed by a second wash at 65°C in the presence of 0.1xSSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to a given gene sequence are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at 42°C, 6xSSC, and 1% SDS; and a second wash at 50°C, 6xSSC, and 1% SDS.
2. This is information that represents background knowledge for any skilled person in the present technical field, and it outlined in such commonly used laboratory manuals such as Ausubel *et al.* (Current Protocols in Molecular Biology, John Wiley & Sons, 1989).

Declared at: Imperial College, London UK
Place
26th July 2005
Date P. Reeves

Before me,

1/11/05

Name: BRIAN GEOFFREY SPRATT FR S
Qualification: PROFESSOR OF MICROBIOLOGY
Address: IMPERIAL COLLEGE LONDON, ST MARY'S CAMPUS, LONDON W2 1PG.
[Full name, qualification and address of person before whom the declaration is made (in printed letters)]

CURRICULUM VITAE

Peter Richard REEVES

Born 17th December 1934.

QUALIFICATIONS AND HONOURS

B.Sc. Hons 2(1) in Zoology, University of London (University College, 1953-56).

PhD. University of London (Wright-Fleming Institute, St. Mary's Hospital, 1956-59).

Fellow Australian Academy of Science (Elected 2000)

APPOINTMENTS

1. Guest worker at the Haffkine Institute, Bombay, India - January - July 1960
2. Research Fellow in the Microbiology Department, University of Adelaide, initially as a Wellcome Trust Research Fellow (January 1961 - December 1962), then a University of Adelaide Postdoctoral Fellow (January 1963 - December 1964).
3. Lecturer, Senior Lecturer & Reader in the Microbiology Department, The University of Adelaide (January 1965-August 1985).
4. Professor and Head of Microbiology Department, The University of Sydney (August 1985-2003).
5. Professor and Head of Microbiology Discipline, School of Molecular and Microbial Biosciences, The University of Sydney (2003- 2005).

GRADUATE STUDENTS

29 students who studied under my supervision have been awarded the Ph.D degree at either the University of Adelaide or University of Sydney and two were awarded the M.Sc. degree. All postgraduate students have gained the degree they sought. Many have taken postdoctoral positions around the world including at Max Planck Institutes, Berkeley, Princeton, Stanford and Oxford. In later careers they have done well in Universities, CSIRO, Government and Industry in Australia and overseas. Two have chairs, three are readers or associate professors and two are lecturers: others are public servants, in CSIRO or in Industry and one is a patent attorney.

There are currently 5 Ph.D. students in the laboratory.

GRANTS

ARC grants

I have had ARC grant support continuously since 1969, totaling over \$3.5m.

NH&MRC grants

I have held NH&MRC grants for most of the time since 1984. The grants were for two or three years each, and totaled \$1.7m.

NBP, GIRD and Industry grants

I have undertaken research on several commercial projects, all of which involved interesting science as well as commercial potential.

I was one of the founders of ENTEROVAX, a company established and owned jointly by The University of Adelaide and Faulding Co Ltd. to develop vaccines using recombinant DNA techniques. The research undertaken was funded by a National Biotechnology Program grant of \$860,000 and additional funds from F. H. Faulding Co Ltd., starting in 1984. The project was successful in developing a potential cholera vaccine, which had an encouraging human challenge trial in the USA, and in Adelaide there were successful pig challenge trials of potential *E. coli* scours vaccines. A second human challenge trial was being arranged after improvements to the vaccine but this and further pig trials were halted when Faulding Co Ltd. came under takeover threat and had a change of policy.

Dr Ian Holmes and I worked together for some years on a vaccine for rotavirus. The target vaccine was to express rotavirus antigens on the surface of a Salmonella vaccine strain, and be achieved by fusing rotavirus and bacterial outer membrane genes to give an outer membrane protein with rotavirus antigens. The project combined Dr Holmes' skills with rotavirus and mine with recombinant DNA and outer membrane proteins in particular. The project started in 1987 with Wallace Biomedical Pty Ltd. which provided funds for 1987 and 1988 (total \$428,156 to my group) before it failed as a Company in 1989, but after a delay the project was continued with Cyanamid Australia Pty Ltd and a GIRD grant (1993-1996). As before the work and funds were divided about equally between the two groups with a total of \$700,000 spent in Sydney on my part of the project. Again the project was successful in achieving its aim, in this case making and testing a range of constructs and finally expression of specific rotavirus antigens on the surface of Salmonella, but in 1993 the Cyanamid parent company in the USA was taken over by American Home Products, which decided not to continue the project.

I have also undertaken work on development of diagnostics and vaccines for *E. coli* O111 and O157 strains funded by Bioproperties Pty Ltd. (Total funding \$153,739 plus overheads since 1996). This work has been quite successful with patents taken out and arrangements made with Perkin Elmer for development of diagnostic products.

RESEARCH WORK

PhD. Genetics of *E. coli* K12 working on Hfr strains and the nature of recombination.

Adelaide and Sydney

1. Chemical nature, genetics and mode of action of colicins.

Bacteriocins are antibiotic-like proteins, which are remarkable in that they act in general against other bacteria of the same or closely related species. Those of *Escherichia coli* and its relations are called colicins, and I worked on their classification, interaction with sensitive cells, the nature of resistant mutants and mode of action.

During this period we showed that colicins fall into 2 distinct groups - A and B, which interact with cells through quite different pathways. This and other work of ours and others was summarised in a monograph in 1972, and I continued to work on colicins until about 1976.

2. Surface proteins of *E. coli*.

Most colicin resistant mutants have altered colicin binding properties and many phage resistant mutants were known to have lost a surface receptor and to also be resistant to colicins. A

comprehensive study of such mutants lead us into a study of bacterial surface proteins. We became one of the groups characterising the major surface proteins of outer membrane. Our major interest was in defining the genes, looking at regulation and the function of the proteins. This general area of work continued until about 1987.

3. Genetics and Evolution of O antigens and other repeat unit polysaccharides

The outer membrane of gram negative bacteria characteristically has, as a major surface antigen, a polysaccharide known as the O antigen. The structure of this O antigen varies among strains, and over 50 forms are known for *Salmonella* and over 160 for *Escherichia coli*. Each has an oligosaccharide of 2-6 sugars which is polymerised to give a "repeat unit polysaccharide". The development of molecular genetic techniques allows analysis of the genetic basis of the phenomenon. We have cloned and sequenced the loci which determine the O antigen and some capsules from several *Salmonella*, *E. coli* and *Yersinia pseudo tuberculosis* strains, including characterisation of the genes for the several biosynthetic pathways involved. This work has lead to an understanding of the origins of the variation in O antigens, which are shown to be encoded by gene clusters which have been assembled from modules to put together the biosynthetic pathways needed for the specific repeat unit polysaccharide. These then are transferred within and between the species to give the enormous variety one finds. We have described examples of recombination within a species to generate a new O antigen, transfer of genes from a capsule gene cluster to an O antigen gene cluster and transfer between species. In general the time frames for evolution of new forms and between species is very long, but movement within species more frequent.

Indeed a comparative analysis indicates that the O antigen forms are much older than the species themselves, and it seems that bacteria acquire new surface antigens by lateral transfer from other, often quite unrelated, species. We are currently exploring the extent of this unexpected finding, which has considerable implications for bacterial evolution.

In the techniques area we were the first to take to sequencing complete O antigen clusters as a single project, which soon became routine but time consuming. We have now adapted for our needs the scale up techniques and robotics which were developed for bacterial genome sequencing, and can now PCR and sequence and analyse a complete 10-15 kb gene cluster within weeks.

4. Bacterial population studies

Population genetics of bacteria lagged behind that of "higher" organisms. Work from several labs has established new methods for study, all involving molecular genetics. We have now been working in the area for several years and are making a contribution. We have published on the population structure of Sonnei, the development of the 7th pandemic clone of *Vibrio cholerae* since its first appearance in 1961, the transfer within *Salmonella* of O antigen gene clusters, the high contribution of lateral gene transfer to variation within bacterial species (20 % of genes in *Salmonella* subspecies 1 are not present at all in subspecies 5), and the relationship of pathogenic and commensal *E. coli* strains (including Shigella).

5. Oral Vaccines

In the early 1980's a group in the Microbiology Dept, University of Adelaide, embarked on a cholera vaccine project. The project obtained government support and a company, Enterovax Pty Ltd., jointly owned by Faulding Pty Ltd and the University of Adelaide was set up to own and operate the project. The O antigen genes of *V. cholera* were cloned and expressed in the *Salmonella* typhoid vaccine strain ty21a, and gave encouraging results in a human challenge trial in Baltimore. There were many people involved in the project, which at the scientific level was successful, but nonetheless was discontinued when funding was not found for the next stage.

I am currently an investigator with Dr I. Holmes and Prof Peter Coloe in research program on Antigenic expression of viral protein antigens on *Salmonella* bacterial vectors which was until

recently supported by a GIRD grant. The industrial partners were Cyanamid Australia Pty. Ltd (taken over by American Home Products) and Bioproperties Pty Ltd. This work is looking for a new industrial partner. The aim is to develop generic methods for expression of viral and other proteins on the surface of bacterial cells using rotavirus VP7 as a model. The virus and vector chosen are appropriate for development of a porcine oral vaccine for rotavirus and further development of a similar human rotavirus vaccine. The work achieved its goals for the GIRD grant period.

AUSTRALIAN GENOMIC INFORMATION SERVICE (AGIC)

I was instrumental in setting up in 1990 the Australian National Genomic Information Service (ANGIS) and AGIC (Australian Genomic Information Centre), the University of Sydney Centre which runs the service. Dr. Alex Reisner had the expertise to develop and run the service and I convened the group which made the submission in 1990 to DITAC (Department of Industry, Technology and Commerce) which was seeking proposals to establish a National Genomic Information Service. The submission was successful and the University established AGIC, of which I have been Executive Director from 1991 until now. As convenor of the initial group and since 1991 as Executive Director I have been responsible, with the appointed Head (Dr. Alex Reisner and more recently Dr. Tim Littlejohn) for negotiating the constitution of AGIC, grant funding, and many policy matters. We provide in ANGIS a very comprehensive set of sequence, gene and genome databases plus a suite of programs for analysis. In only a few years ANGIS has grown to be very well known amongst Australian Molecular Biologists: it continues to develop and innovate and a spin-off company, eBioinformatics, is marketing a revised version overseas as BioNavigator.

ADMINISTRATION

Head of Microbiology Department, University of Sydney (1985 to 2002)

Head of Microbiology Discipline, School of Molecular and Microbial Biosciences, University of Sydney (2002 - present)

Pro Dean of the Faculty of Agriculture, University of Sydney (December 1988 to February 1997).

ARC Collaborative Grants Committee (1992 to 1993)

Executive Director of AGIC (1991 to present)

University Committees including:

Biosafety Committees at University of Adelaide and University of Sydney,

Coordinating Committee (University of Adelaide) which advised on coordination of Faculty decisions, resource implications and university policy,

University Promotion Committees,

Chair appointment Committees at La Trobe University, University of Melbourne, University of Queensland and University of Sydney.

SERVICE TO THE COMMUNITY

Foundation member of Nature Conservation Society of South Australia in 1962. Treasurer 1962 to 1972 and President from 1974 to 1979.

Member of the Management Committee of the Total Environment Centre, Sydney, since 1986.

These two organisations have been, in their respective States, among the most effective non-Government organisations in bringing about change in environmental matters. They both work effectively with Government while retaining independence and respect.

Elected Council member from South Australia of Australian Conservation Foundation (ACF) 1974 to 1985.

Appointed member of Environment Protection Council (EPC) of South Australia 1978 to 1985. EPC was a statutory body, which advised the Premier on environmental matters, either on request or of its own volition, often after commissioning reports. It comprised Heads of relevant Government Departments and appointed non-government members. In this capacity I was occasionally on South Australian Government delegations at inter-Government meetings.

PUBLICATIONS

1. Reeves, P., *Role of Hfr mutants in F^+ x F^- crosses in E. coli K12*. Nature, 1960. **185**: p. 265-266.
2. Reeves, P., *Preparation of a substance having colicin F activity from Escherichia coli CA42*. Aust. J. exp. Biol. & med.Sci., 1963. **41**: p. 163-170.
3. Krishnapillai, V., P.R. Reeves, and D. Rowley, *Genetic and immunological observations on the virulence of Salmonella typhimurium for mice*. Aust. J. exp. Biol. & med. Sci., 1963. **41**: p. 61-72.
4. Reynolds, B.L. and P. Reeves, *Some observations on the mode of action of colicin F*. Biochem. Biophys. Res. Comm., 1963. **11**: p. 140-145.
5. Reeves, P., *The adsorption and kinetics of killing by colicin CA42-E₂*. Aust. J. Exp. Biol. & Med.Sci., 1965. **43**: p. 191-200.
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7. Reeves, P., *Mutants resistant to colicin CA42-E₂. Cross resistance and genetic mapping of a special class of mutants*. Aust. J. Exp. Biol. & Med. Sci., 1966. **44**: p. 301-316.
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14. Skurray, R.A. and P. Reeves, *Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12*. Journal of Bacteriology, 1973. **113**: p. 58-70.
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- Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J. Bacteriol., 1974. 119: p. 726-735.
18. Reeves, P. and N. Willets, *Plasmid specificity of the origin of transfer of sex factor F*. J. Bacteriol., 1974. 120: p. 125-130.
 19. Hancock, R.E.W. and P. Reeves, *Bacteriophage resistance in E. coli K-12: general pattern of resistance*. J. Bacteriol., 1975. 121: p. 983-993.
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 24. Hancock, R.E.W., J.K. Davies, and P. Reeves, *Cross resistance between bacteriophages and colicins in E. coli K-12*. J. Bacteriol., 1976. 126: p. 1347-1350.
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Molecular Biology Techniques Manual

Third Edition

Edited by:

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PCR PRIMER DESIGN AND REACTION OPTIMISATION

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Biology, University of Cape Town

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Contents

- Factors Affecting the PCR
 - Denaturing Temperature and Time
 - Annealing Temperature and Primer Design
 - Primer Length
 - Degenerate Primers
 - Elongation Temperature and Time
 - Reaction Buffer
 - Cycle Number
 - Nested Primer PCR
 - Labelling of PCR products with digoxigenin-11-dUTP
 - Helix Destabilisers / Additives
 - Useful Universal cDNA PCR Primer
 - A simple set of rules for primer sequence design
 - REFERENCES
-

Factors Affecting the PCR:

Denaturing Temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (**G to C, and A to T or U**) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing - if it is not single-stranded already, like **most RNA viruses** - by heating it to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than **150mM NaCl**, the melting temperature is generally less than 100°C - which is why PCR works with **denaturing temperatures of 91-97°C**.

A more detailed treatment of annealing / hybridisation is given in an accompanying page, together with explanations of calculations of complexity, conditions for annealing / hybridisation, etc.

Taq polymerase is given as having a half-life of **30 min** at **95°C**, which is partly why one should not do more than about **30 amplification cycles**: however, it is possible to **reduce the denaturation temperature** after about 10 rounds of amplification, as the **mean length of target DNA is decreased**: for templates of **300bp or less**, denaturation temperature may be reduced to as low as **88°C for 50% (G+C)** templates (Yap and McGee, 1991), which means one may do as many as **40 cycles** without much decrease in enzyme efficiency.

"**Time at temperature**" is the main reason for denaturation / loss of activity of Taq: thus, if one reduces this, one will **increase the number of cycles that are possible**, whether the temperature is reduced or not. Normally the denaturation time is **1 min at 94°C**: it is possible, for short template sequences, to **reduce this to 30 sec or less**. Increase in denaturation temperature and decrease in time may also work: Innis and Gelfand (1990) recommend **96°C for 15 sec**.

Annealing Temperature and Primer Design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature of a NA duplex increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the T_m is

$$T_m = 4(G + C) + 2(A + T)^{\circ}\text{C}.$$

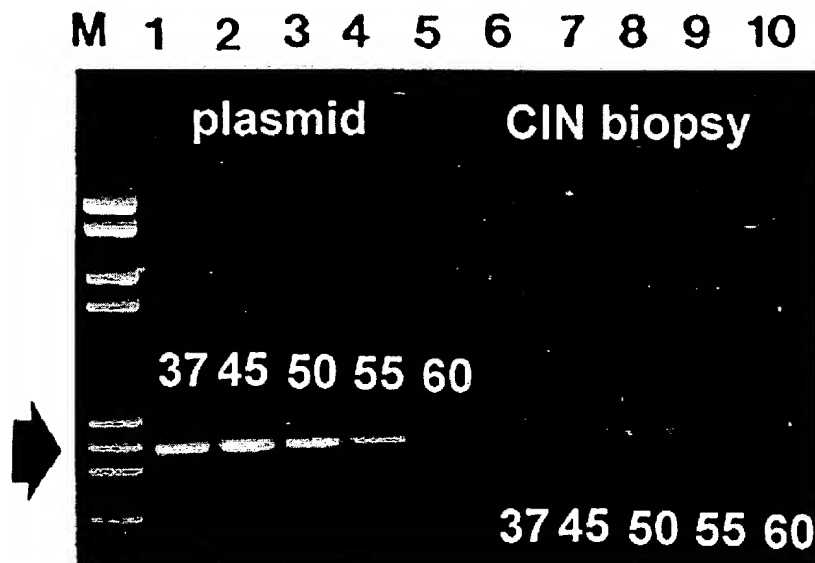
Thus, the annealing temperature chosen for a PCR depends **directly on length and composition** of the primer(s). One should aim at using an annealing temperature (T_a) about **5°C below the lowest T_m of their pair of primers to be used** (Innis and Gelfand, 1990). A more rigorous treatment of T_a is given by Rychlik *et al.* (1990): they maintain that **if the T_a is increased by 1°C every other cycle, specificity of amplification and yield of products <1kb in length are both increased**. One consequence of having too low a T_a is that one or both primers will **anneal to sequences other than the true target**, as internal single-base mismatches or partial annealing may be tolerated: **this is**

fine if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target.

A consequence of too high a T_a is that **too little product will be made**, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different T_a s **may never give appreciable yields of a unique product**, and may also result in inadvertent "asymmetric" or single-strand amplification of the most efficiently primed product strand.

Annealing does not take long: most primers will anneal efficiently in 30 sec or less, unless the T_a is too close to the T_m , or unless they are unusually long.

An illustration of the effect of annealing temperature on the specificity and on the yield of amplification of *Human papillomavirus type 16* (HPV-16) is given below (Williamson and Rybicki, 1991: J Med Virol 33: 165-171).



Plasmid and biopsy sample DNA templates were amplified at different annealing temperatures as shown: note that while plasmid is amplified from 37 to 55°C, HPV DNA is only specifically amplified at 50°C.

Primer Length

The optimum length of a primer depends upon its (A+T) content, and the T_m of its partner if one runs the risk of having problems such as described above. Apart from the T_m , a prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. ([See hybridn.doc](#)).

For example, there is a $\frac{1}{4}$ chance (4^{-1}) of finding an A, G, C or T in any given DNA

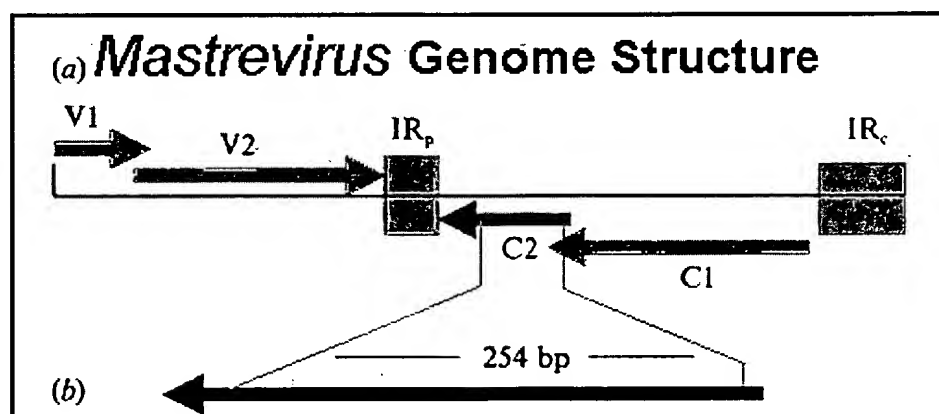
sequence; there is a $1/16$ chance (4^{-2}) of finding any dinucleotide sequence (eg. AG); a $1/256$ chance of finding a given 4-base sequence. Thus, a **sixteen base sequence will statistically be present only once in every 4^{16} bases (=4 294 967 296, or 4 billion)**: this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater-than-17-base oligonucleotide with its target sequence is an extremely sequence-specific process, far more so than the specificity of monoclonal antibodies in binding to specific antigenic determinants. Consequently, **17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants**. Too long a primer length may mean that even high annealing temperatures are not enough to prevent mismatch pairing and non-specific priming.

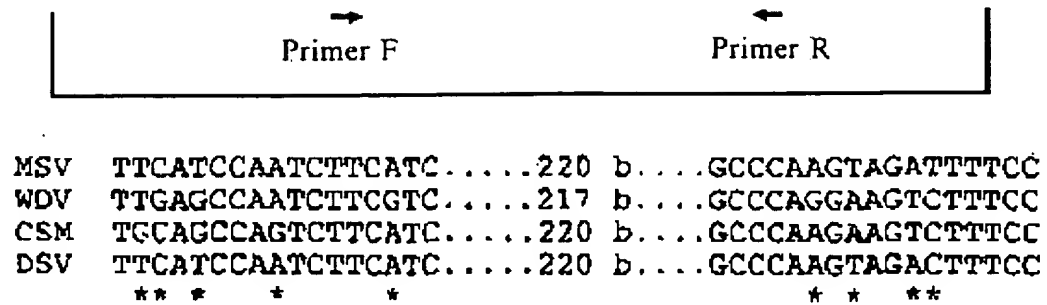
Degenerate Primers

For amplification of cognate sequences from different organisms, or for "evolutionary PCR", one may increase the chances of getting product by designing "degenerate" primers: these would in fact be a set of primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences. For example, Compton (1990) describes using 14-mer primer sets with 4 and 5 degeneracies as forward and reverse primers, respectively, for the amplification of glycoprotein B (gB) from related herpesviruses. The reverse primer sequence was as follows:

TCGAATTCNCCYAAYTGNCNT

where Y = T + C, and N = A + G + C + T, and the 8-base 5'-terminal extension comprises a *EcoRI* site (underlined) and flanking spacer to ensure the **restriction enzyme can cut the product** (the New England Biolabs catalogue gives a good list of which enzymes require how long a flanking sequence in order to cut stub ends). **Degeneracies obviously reduce the specificity of the primer(s)**, meaning mismatch opportunities are greater, and background noise increases; also, **increased degeneracy means concentration of the individual primers decreases**; thus, greater than 512-fold degeneracy should be avoided. However, I have used primers with as high as 256- and 1024-fold degeneracy for the successful amplification and subsequent direct sequencing of a wide range of *Mastreviruses* against a background of maize genomic DNA (Rybicki and Hughes, 1990).

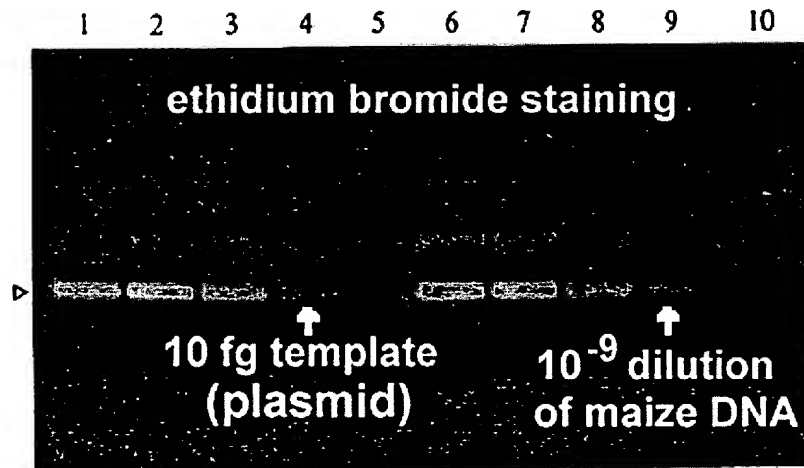




Primer sequences

F: 5' - T**A*CCA*TCTTC*TC - 3'
 R: 5' - GGAAA**CT*C*TGGGC - 3'

Primer sequences were derived from multiple sequence alignments; the mismatch positions were used as 4-base degeneracies for the primers (shown as stars; 5 in F and 4 in R), as shown above. Despite their degeneracy, the primers could be used to amplify a 250 bp sequence from viruses differing in sequence by as much as 50% over the target sequence, and 60% overall. They could also be used to very sensitively detect the presence of *Maize streak virus* DNA against a background of maize genomic DNA, at dilutions as low as $1/10^9$ infected sap / healthy sap (see below).



Some groups use deoxyinosine (dI) at degenerate positions rather than use mixed oligos: this base-pairs with any other base, effectively giving a four-fold degeneracy at any position in the oligo where it is present. This lessens problems to do with depletion of specific single oligos in a highly degenerate mixture, but may result in too high a degeneracy where there are 4 or more dIs in an oligo.

Elongation Temperature and Time

This is normally 70 - 72°C, for 0.5 - 3 min. Taq actually has a specific activity at 37°C which is very close to that of the Klenow fragment of *E coli* DNA polymerase I, which accounts for the apparent paradox which results when one tries to understand how primers which anneal at an optimum temperature can then be elongated at a considerably higher temperature - the answer is that elongation occurs from the moment of annealing, even if this is transient, which results in considerably greater stability. At around 70°C the activity is optimal, and primer extension occurs at up to 100 bases/sec. About 1 min is sufficient for reliable amplification of 2kb sequences (Innis and Gelfand, 1990). Longer products require longer times: **3 min is a good bet for 3kb and longer products.** Longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration (>1nM), and when dNTP and / or primer depletion may become limiting.

Reaction Buffer

Recommended buffers generally contain :

- 10-50mM Tris-HCl pH 8.3,
- up to 50mM KCl, 1.5mM or higher MgCl₂,
- primers 0.2 - 1µM each primer,
- 50 - 200µM each dNTP,
- gelatin or BSA to 100µg/ml,
- and/or non-ionic detergents such as Tween-20 or Nonidet P-40 or Triton X-100 (0.05 - 0.10% v/v)

(Innis and Gelfand, 1990). Modern formulations may differ considerably, however - they are also generally proprietary.

PCR is supposed to work well in reverse transcriptase buffer, and vice-versa, meaning 1-tube protocols (with cDNA synthesis and subsequent PCR) are possible (Krawetz *et al.*, 19xx; Fuqua *et al.*, 1990).

Higher than 50mM KCl or NaCl inhibits Taq, but some is necessary to facilitate primer annealing.

[Mg²⁺] affects primer annealing; T_m of template, product and primer-template associations; product specificity; enzyme activity and fidelity. Taq requires *free* Mg²⁺, so allowances should be made for dNTPs, primers and template, all of which chelate and sequester the cation; of these, dNTPs are the most concentrated, so [Mg²⁺] should be 0.5 - 2.5mM *greater* than [dNTP]. **A titration should be performed with varying [Mg²⁺] with all new template-primer combinations,** as these can differ markedly in their requirements, even under the same conditions of concentrations and cycling times/temperatures.

Some enzymes do not need added protein, others are dependent on it. Some enzymes work markedly better in the presence of detergent, probably because it prevents the natural tendency of the enzyme to aggregate.

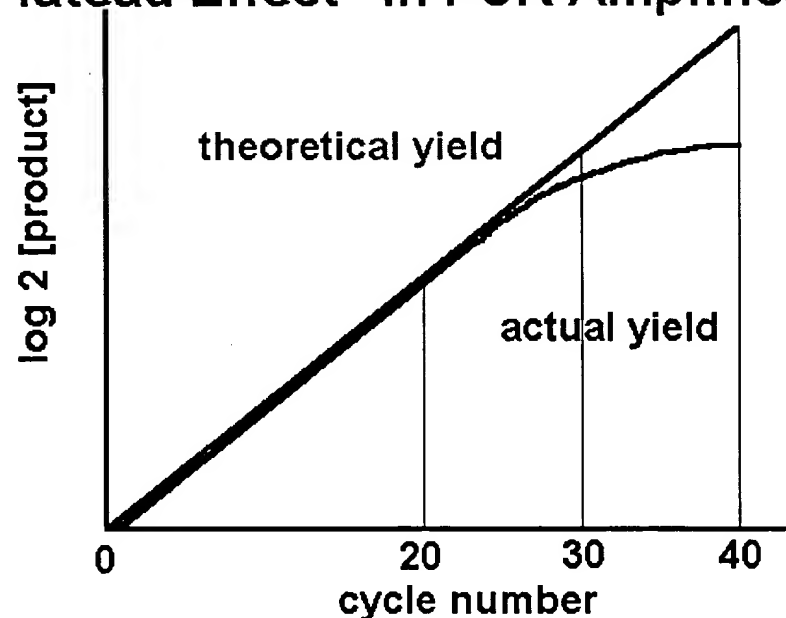
Primer concentrations should not go above 1µM unless there is a high degree of degeneracy; 0.2µM is sufficient for homologous primers.

Nucleotide concentration need not be above 50uM each: long products may require more, however.

Cycle Number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: Innis and Gelfand (1990) recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify 3×10^5 molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect*, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs - former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).

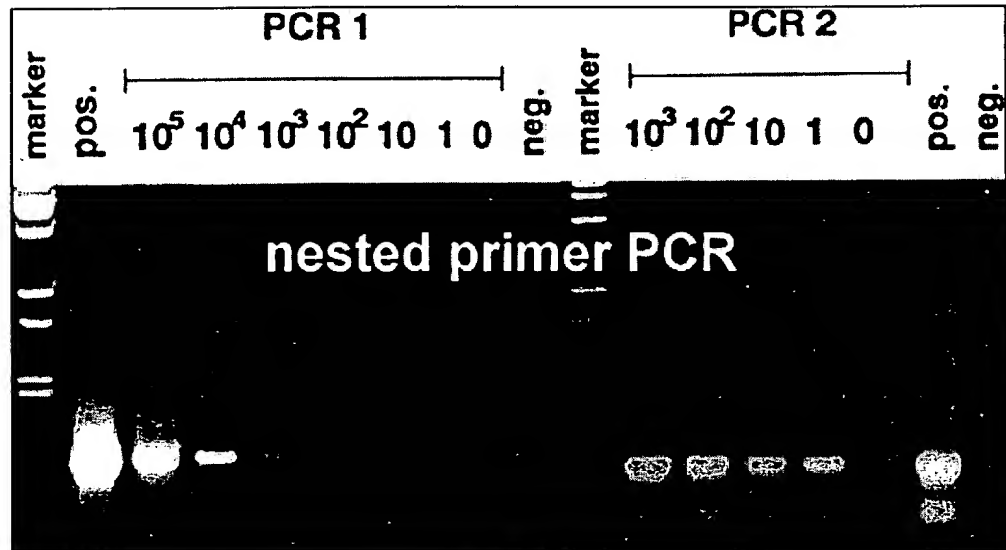
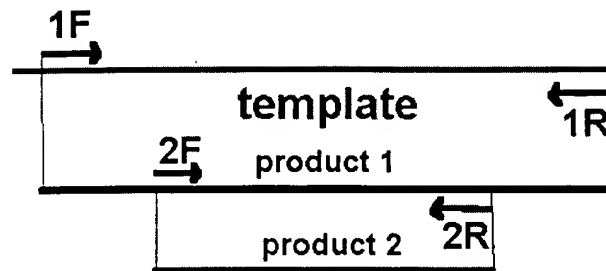
"Plateau Effect" in PCR Amplification



If desired product is not made in 30 cycles, take a small sample (1ul) of the amplified mix and re-amplify 20-30x in a new reaction mix rather than extending the run to more cycles: in some cases where template concentration is limiting, this can give good product where extension of cycling to 40x or more does not.

A variant of this is **nested primer PCR**: PCR amplification is performed with one set of primers, then some product is taken - with or without removal of reagents - for re-amplification with an internally-situated, "nested" set of primers. This process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second. This is illustrated below:

NESTED PRIMER PCR:



This gel photo shows the effect of nested PCR amplification on the detectability of *Chicken anaemia virus* (CAV) DNA in a dilution series: the PCR1 just detects 1000 template molecules; PCR2 amplifies 1 template molecule (Soiné C, Watson SK, Rybicki EP, Lucio B, Nordgren RM, Parrish CR, Schat KA (1993) Avian Dis 37: 467-476).

Labelling of PCR products with digoxigenin-11-dUTP

(DIG; Roche) need be done only in 50uM each dNTP, with the dTTP substituted to 35% with DIG-11-dUTP. **NOTE:** that the product will have a higher MW than the native product! This results in a very well labelled probe which can be extensively re-used, for periods up to 3 years. See also [here](#).

Helix Destabilisers / Additives

With NAs of high (G+C) content, it may be necessary to use harsher denaturation conditions. For example, one may incorporate up to 10% (w or v/v) :

- dimethyl sulphoxide (DMSO),
- dimethyl formamide (DMF),
- urea
- or formamide

in the reaction mix: these additives are presumed to lower the T_m of the target NA, although DMSO at 10% and higher is known to decrease the activity of Taq by up to 50% (Innis and Gelfand, 1990; Gelfand and White, 1990).

Additives may also be necessary in the amplification of long target sequences: DMSO often helps in amplifying products of >1kb. Formamide can apparently dramatically improve the specificity of PCR (Sarkar *et al.*, 1990), while glycerol improves the amplification of high (G+C) templates (Smith *et al.*, 1990).

Polyethylene glycol (PEG) may be a useful additive when DNA template concentration is very low: it promotes macromolecular association by solvent exclusion, meaning the pol can find the DNA.

cDNA PCR

A very useful primer for cDNA synthesis and cDNA PCR comes from a sequencing strategy described by Thweatt *et al.* (1990): this utilised a mixture of three 21-mer primers consisting of 20 T residues with 3'-terminal A, G or C, respectively, to sequence inside the poly(A) region of cDNA clones of mRNA from eukaryotic origin. I have used it to amplify discrete bands from a variety of poly(A)+ virus RNAs, with only a single specific degenerate primer upstream: the T-primer may anneal anywhere in the poly(A) region, but only molecules which anneal at the beginning of the poly(A) tail, and whose 3'-most base is complementary to the base next to the beginning of the tail, will be extended.

eg:

5'-TTTTTTTTTTTTTTTTTTTTTTTTTTT(A,G,C)-3'

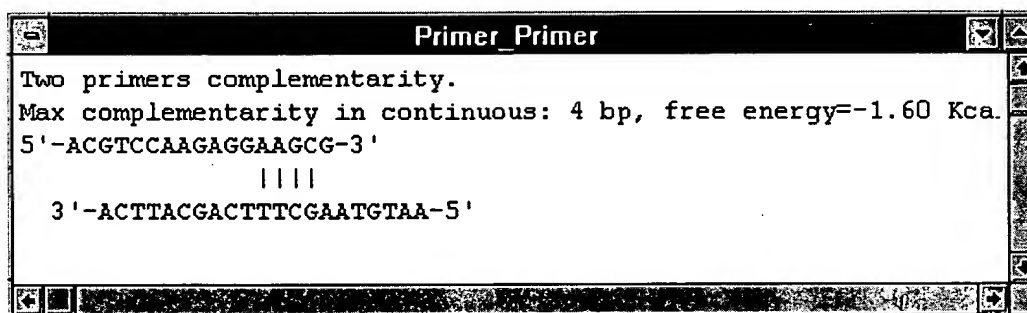
works for amplification of *Potyvirus* RNA, and eukaryotic mRNA

A simple set of rules for primer sequence design is as follows (adapted from Innis and Gelfand, 1991):

1. primers should be 17-28 bases in length;
2. base composition should be 50-60% (G+C);
3. primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;

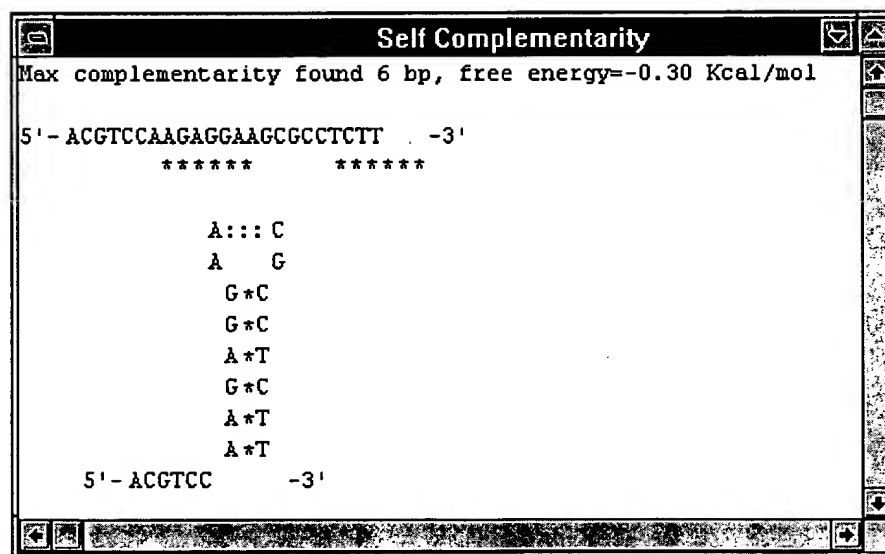
4. Tms between 55-80°C are preferred;
5. runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
6. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product;
7. primer self-complementarity (ability to form 2° structures such as hairpins) should be avoided.

Examples of inter- and intra-primer complementarity which would result in problems:



```

Primer_Primer
Two primers complementarity.
Max complementarity in continuous: 4 bp, free energy=-1.60 Kca.
5'-ACGTCCAAGAGGAAGCG-3'
      ||||
3'-ACTTACGACTTTCGAATGTAA-5'
  
```



```

Self Complementarity
Max complementarity found 6 bp, free energy=-0.30 Kcal/mol
5'-ACGTCCAAGAGGAAGCGCCTCTT-3'
      *****
      A:::C
      A  G
      G*C
      G*C
      A*T
      G*C
      A*T
      A*T
5'-ACGTCC-3'
  
```

Screen shots taken from analyses done using DNAMAN (Lynnon Biosoft, Quebec, Canada).

[Return to PCR Contents Page](#)

[Return to Molecular Biology Methods Manual](#)

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[Return to PCR Contents Page](#)

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